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(54) Title: ASSAYS TO EVALUATE CHEMOTHERAPEUTIC EFFECTIVENESS OF DRUGS

(57) Abstract

In vitro assays which respond to signal pathway modulating compounds in transformed cells are described. By measuring the effect of candidate drugs on specific kinase targets, successful candidates can be screened. Tumor biopsies can be assayed for susceptibility to treatment with a particular drug using similar assays. The VL30 controlled expression of a reporter gene can also be used in screening assays for both inhibitors and stimulators of cell proliferation.

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ASSAYS TO EVALUATE CHEMOTHERAPEUTIC EFFECTIVENESS OF DRUGS

Acknowledgment of Government Support

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Field of the Invention

The invention relates to *in vitro* methods to predict and to monitor the effectiveness of chemotherapeutic agents, such as CAI, that modulate intracellular signal pathways involved in proliferation. CAI is a known chemotherapeutic agent effective only with respect to some cancers.

Background Art

In contrast to many chemotherapeutic agents which work by directly interfering with DNA replication and thus with proliferation of both normal and cancer cells, an alternative group of chemotherapeutic agents targets specific signalling pathways related to proliferation. It appears that normal cells generally have parallel signalling pathways for proliferation, so that inhibition of individual signalling pathways as targeted by this class of drugs can mostly be overcome by normal cells. Tumor cells, however, often are specialized in one particular overexpressed pathway so that successful inhibition of this pathway is specifically effective against cancer cells. This is highly advantageous since the inhibition of replication of normal cells, especially those in the blood marrow, the lining of the GI tract and hair follicles, results in the devastating side-effects of chemotherapy.

An illustrative member of this class of signal pathway modulating agents is described in U.S. patents 4,847,257 and 5,045,543 to Merck & Co., the contents of which are incorporated herein by reference. These patents describe the use of a known class of compounds -- 5-amino or substituted amino, 1,2,3-triazoles in the treatment of psoriasis and cancer. According to the disclosures of the above-referenced patents, the compounds, in addition to being antiproliferative agents, also inhibit the production of a broad array of arachidonate metabolites. A preferred member of this class of compounds is 5-amino-1-(4-[4-chlorobenzoyl]-3,5-dichlorobenzyl)-1,2,3-triazole-4-carboxamide (CAI). This compound is currently in phase I clinical trials as a chemotherapeutic agent.

It has recently been reported that although CAI is nontoxic for the reasons described above, it is not broadly effective as a chemotherapeutic agent generally. It would clearly be advantageous to devise a screening assay to determine which cancers will respond to CAI and which will not so that subjects with tumors susceptible to CAI treatment will be spared the harmful side effects of other drugs, while subjects whose tumors are nonresponsive would be protected from lost time due to ineffective treatment. The present invention relates to such screening tests, applicable not only to CAI, but to signalling pathway inhibitors generally. Assays presently available in the art for predicting the effectiveness of drugs against tumors are notably unsuccessful and cumbersome. All of them require culturing of the tumor cells, a process which is not only time-consuming, but unreliable, since the culture conditions themselves may alter the results of the assay. Assays based on the signaling transduction system as described in the present invention do not require culturing of the tumor cells.

The types of assays used to predict effectiveness of a drug against a particular tumor also provide a means to screen candidate drugs for their ability to inhibit proliferation by interfering with signal transduction. These and similar assays based on cells modified to contain a target expression system can thus also be used to screen candidate drugs.

Disclosure of the Invention

The invention is directed to assays which can be conducted *in vitro* to determine subjects who will and who will not respond to treatment by drugs, such as CAI, that interfere with intracellular signalling pathways and to monitor subjects under treatment for continued effectiveness of the protocol.

The assays do not require culturing of the cells to be tested and utilize straightforward assessment techniques to determine the effect of such drugs. The assays are of two general types: one type relies on assessing systems that are at the end-points of the cell signalling pathways that lead to proliferation, specifically the p34cdc2 kinase system. Because these components are essentially at the convergence of a multiplicity of signalling pathways, modulation, either of them directly, or of their upstream signalling reactions by a drug will result in modulation of these components themselves.

Alternatively, upstream targets for specific antitherapeutic agents, once identified, can be used as the basis for these assays. The present inventors have identified certain upstream indicators which are affected by CAI, including inhibition of the activity of src kinase and the phosphorylation state of the GAP-associated protein p62. Phosphorylation of p62 is associated with increased activation of ras-dependent signalling pathways.

On the other hand, p34cdc2 is a cell cycle associated kinase that plays a key role as an checkpoint kinase in cellular proliferation, specifically at the G2/M transition. Drugs effecting any proliferation-associated signal pathway will have an effect on this end point.

In one aspect, the invention is directed to a method to screen candidate drugs for their ability to inhibit cell proliferation through interaction with intracellular signal pathways in target cells. The method comprises incubating a test sample of the cells in the presence of the candidate and incubating a control sample of the cells in the absence of the candidate. The activity of p34cdc2 kinase is then measured in each of the cell samples and the activities of the p34cdc2 kinase are compared. A diminution of activity of the kinase in the presence as compared to the absence of the candidate indicates the ability of the candidate to inhibit proliferation.

In still another aspect, the invention concerns an alternative method to screen candidate drugs for their ability to stimulate or inhibit cell proliferation in target cells. The method also comprises incubating a test sample of cells in the presence of the candidate and incubating a control sample of cells in the absence of the candidate, and stimulating the cells with at least one known agonist. Gene expression mediated by the VL30 enhancer, as evidenced by a VL30-controlled reporter system in each of the cell samples is measured and compared. A change of the expression in the presence of the candidate as compared to the absence of the candidate indicates the ability of the candidate to impact signal transduction, which ability can then be dissected through additional studies with various agonists using the same VL30 enhancer-controlled reporter system. As shown below, a particular candidate may have different effects depending on whether the cells are treated with the

candidate prior to addition of the agonist or contemporaneously therewith. For a complete profile of a particular candidate drug, it will be useful to measure its effects under both chronic (pretreatment) and acute (simultaneous treatment) conditions.

In another aspect the invention concerns a method to determine whether a drug will or will not function as an antitumor agent with respect to target tumor cells. The method comprises incubating a test sample of a biopsy containing these cells in the presence of the candidate and incubating a control sample of a biopsy containing these cells in the absence of the drug. The activity of p34cdc2 kinase in each of the cell samples is measured and the activities of the p34cdc2 kinase are compared. A diminution of activity of the kinase in the presence of the drug as compared to the absence of the drug indicates the drug will be effective in treatment of the tumor.

If a particular drug is known to effect a component of a signal transduction pathway, this particular component can also be used as the subject of an assay after incubating the tumor cells of the biopsy in the presence and absence of the drug. Although this assay requires prior information as to a component of the signalling pathways which is directly effected by a particular drug (or is downstream in the signalling pathway from the drug's direct target) this approach still retains the advantages of simplicity of performance and lack of necessity for growing the cells in culture.

Brief Description of the Drawings

Figure 1 shows the effect of CAI treatment on v-src kinase activity.

Figure 2 is a histogram showing the concentration-dependent ability of SKF96365 to inhibit the src kinase.

Figure 3 shows the effect of CAI and SKF96365 on kinase activity of p34cdc2.

Figure 4 shows the effect of CAI on expression of CAT of in Rat1 cells modified to contain the VL30 enhancer-controlled expression system under acute exposure conditions.

Figure 5 shows the effect of pretreatment with CAI at various times prior to agonist stimulation on the expression of CAT in cells modified to contain the VL30 system.

Figure 6A and 6B show the effect of 24 hour pretreatment with CAI and SKF on the level of CAT expression in response to various agonists used as stimulants.

Modes of Carrying Out the Invention

The invention focuses on the complex array of signalling pathways used by cells to promote proliferation and on the transcription control elements modulated as a result of the operation of these pathways. Two end-point systems and at least one upstream component are employed in the methods of the present invention.

One end-point system uses p34cdc2 activity as an indicator. The cyclin-dependent kinase, p34cdc2, must be present in the active form in order for the cells to undergo mitosis (Pines, J. *et al*. New Biol (1990) 2:389-401; Solomon, M.J. *et al*. Cell (1990) 63:1013-1024). The activity of p34 as a kinase is dependent on its physical association with cyclin B. The kinase activity of the p34 protein is inactivated by phosphorylation at tyrosine-15 and threonine-14, but is activated when these sites are dephosphorylated and the threonine at position 161 is phosphorylated. It is thus possible to prepare antibodies to both the inactivated and activated forms of p34 where the antibodies are specifically immunoreactive

with the epitopes containing the appropriate phosphorylation status.

P34cdc2 represents an end-point of a multiplicity of intracellular signalling pathways which converge to activate or inactivate this kinase. Therefore, cell proliferation inhibitors which modulate upstream proliferation associated signal transmission events, as well as those that modulate the activity of p34cdc2 directly, will result in an apparent modulation of p34cdc2 activity. This makes the activity of this kinase a suitable target both for screening candidate drugs which act on cell proliferation signalling pathways at any level and as a diagnostic for determining whether or not a particular tumor contains a crucial signalling pathway that can be disrupted by a proposed therapeutic protocol. Successful results of inhibition of an extant signalling pathway, even though upstream of the p34cdc2 kinase component, will be reflected in the activity levels of this kinase.

Since p34cdc2 kinase is critical for mitosis, inhibition of this kinase activity will result in inhibition of proliferation in transformed cells as reflected, for example, in a decrease in labeled thymidine uptake, a decrease in the ability to grow in soft agar and a diminution of tumorigenicity in nude mice. Assays for these effects correlating to diminished p34 kinase activity confirm the crucial role of p34cdc2 in cell proliferation of tumors and other transformed cells.

As shown in Example 5 of the present application, the assay of the invention gives excellent correlation with traditional assay and is effective in evaluating a variety of candidate drugs including those that stimulate and those that inhibit cell growth.

The second end-point assay involves the use of the VL30 enhancer which is responsive to proliferation-associated transcription factors. This enhancer can be

incorporated into a model expression system containing a reporter gene which provides a measure for activation of the VL30 enhancer by agonists of cell proliferation or for inactivation by proliferation antagonists. Such a construct utilizes the VL30 enhancer, the thymidine kinase promoter, and a reporter gene such as chloramphenicol acetyl transferase (CAT). Other reporter genes, such as β-galactosidase or luciferase can also be used. The reporter gene is used as an indicator of the level of expression which, in turn, is an indicator of stimulation of, or inhibition of, the enhancer. Since the enhancer responds to transcription factors activated by signals associated with cell proliferation, this system provides a means to assess the ability of a candidate drug to effect proliferation in cells that contain such transcription factors. This system has been described in detail by Rodland, K.D. *et al.* Mol Endocrinol (1993) 7:787-796, incorporated herein by reference.

The assay is conducted by measuring the effect of a candidate compound on the expression of the reporter gene stimulated by known at least one agonist of the enhancer. Such agonists include, for example, thapsigargin (thapsi or TG), epidermal growth factor (EGF), and phorbolacetate (TPA). The candidate compound will be added at an appropriate time relative to the stimulation of expression by the agonist, depending on whether an "acute" or "chronic" effect is to be measured. Since the synthesis of the reporter protein to detectable levels through the mediation of the VL30 enhancer in this reporter system takes approximately 4 hours, "chronic" effects of the candidate are tested by pretreating the cells with the candidate so that the interaction between the candidate and factors influencing the enhancer can take place. Suitable pretreatment times range from 4-24 hours or longer. On the other hand, if "acute" effects are to be measured, the candidate

compound is added simultaneously with the agonist, or very close in time thereto.

The VL30 enhancer is a murine retroviral element that is rapidly induced in response to a variety of proliferative stimuli, is superinduced in the presence of protein synthesis inhibitors, and is overexpressed in both virally and chemically transformed cells. To employ this system for screening assays, any convenient recombinant host cells modified to contain the VL30/reporter gene expression system can be used as the assay system. The cells are stimulated with an agonist to effect expression of the VL30 enhancer to produce the product of the reporter gene in the presence and absence of the candidate. Depending on whether the effect to be measured is the acute or chronic effect of the candidate, the candidate is added either along with the agonist or several hours before. The level of reporter gene is measured at a suitable time after stimulation with the agonist, typically about 4 hours. If the acute effect of the candidate is to be measured, the candidate compound is added to the cells generally simultaneously with the agonist. If the chronic effect of the candidate is to be measured, typically the candidate is added to the cells 4-24 hours or longer before addition of the agonist. The assay method used for the assessment of expression of the reporter gene depends, of course, on the nature of the reporter gene itself, and standard assays suitable for the protein products will be known to those of ordinary skill.

It will be apparent that if this assay is to be used in connection with predicting therapeutic effects of drugs on particular tumors, it would be necessary first to modify the tumor cells to contain this system by suitable transformation techniques prior to conducting the assays for the effect of the drug. In general, this modification will be transient. As this is an extra step in performing clinical assessments of this sort, use of

the VL30-based assay system in this context is clearly less advantageous than using the p34cdc2 kinase system as the focus of the assay. However, with respect to screening for candidate drugs, standard recombinant cell lines stably transformed with the expression system can be used as reagents. Moreover, the assay system can be easily automated and adapted to nonradiometric assay methods.

Nevertheless, the results are not as straightforward as those obtained using p34cdc2, since the influence of various signal pathway components on this enhancer is complex; further studies taking account of these pathways may be needed to interpret the results. On the other hand, the VL30 assay system can be used to detect both growth stimulatory and growth inhibitory candidates; growth stimulatory candidates will stimulate VL30 controlled expression; growth inhibitory compounds will inhibit. Generally, stimulation is often observed more easily during acute exposure and inhibition is observed more easily during chronic exposure. For example, CAI increases VL30 expression during an acute exposure (i.e., when the CAI is added along with an agonist), but when the cells containing the VL30 system are pretreated for 4-16 or 24 hours before agonist is added, expression is inhibited. Some compounds, however, exhibit an inhibitory effect when acute exposure conditions are employed. Thus, any particular candidate should be tested under both exposure conditions to obtain an accurate assessment of its activity.

Since drug treatment of tumors would inherently be chronic, the chronic effects will be more relevant to the biological effects of the drug. In this regard, it is important to expose the cells to the candidate drug for a sufficiently long time to obtain the effect of chronic exposure.

P34cdc2 is an end-point marker for many cell proliferation signal pathway modulators and the VL30

reporter system is also responsive to upstream signalling steps. These assays therefore require no knowledge of the actual target for a drug. It will also be useful, however, to assess tumors for their susceptibility to proposed protocols by using as assay targets particular upstream signalling factors which are known to be effected by the drug in question. This is exemplified by the demonstrated effect of CAI and SKF96365 on the kinase activity of the src protein. Since the inventors herein have established that these compounds effect inhibition of src kinase activity, tumor biopsies can be assessed to predict the effectiveness of these drugs as antitumor treatments by measuring their effect on the src kinase activity of the tumor cells in the biopsy. (Both the oncogene (v-src) and the normal (c-src) forms are responsive.) This is a more advantageous approach than measuring the effect of the drug directly on cell proliferation or measuring its cytotoxic effect on cell culture, since it involves an *in vitro* short-term assay which does not require culturing and growth of the cells from the biopsy. This inherent advantage is characteristic of all of the assay methods and screening methods herein. However, the assays involving distal end-points, of course, have the additional advantage of being more universal in scope.

Assays on tumor Biopsies

Thus, all of the assays described herein, whether focused on end-point signal junctions or on upstream components of the signalling pathway identified as effected by a particular candidate drug share the advantage of simplicity and economy. None requires the culturing of tumor cells. This provides a distinct advantage over existing assays and offers the possibility of making such prescreening of tumor biopsies a routine procedure in the process of designing an initial therapeutic protocol and in deciding whether to maintain

a protocol that is currently being applied to a particular patient.

This is not the case now due to the complexity of pretreatment screening of tumors. Although chemotherapeutic treatment is notoriously distasteful, and although it is well understood that no single chemotherapeutic protocol is generically effective against tumors, or even permanently effective against any tumor, no reasonable assays have been available to predict in advance whether a particular protocol or the continuation of a particular protocol will or will not be effective. The currently available assays are based on the clonogenic growth of dissociated tumor cells and thus require subcultivation of the tumor tissue over a period of days or weeks. Furthermore, only 40-70% of tumors subjected to clonogenic assays give results that can be evaluated. Cytotoxicity assays, which do not require culturing, still retain the time and plating efficiency constraints associated with these culture-based methods. There are no currently available *in vitro* assays which simply require measurement of enzyme activity or other simple determination on cellular extracts or a simple histological test. Because the assays of the invention focus on particular components of the signalling systems that regulate proliferation, these components can be measured directly, either by standard *in vitro* assays or by histological staining without the necessity for subculturing the cells, as will further be described below.

The assessment of activities of various enzymes in cellular extracts can be conducted in standard protocols for such enzyme assays; particular assays for kinase activity have been described; however, alternative ways of measuring such kinase activities can also be employed. For example, rather than measuring incorporation of radioactive phosphorus into substrate or incorporation of labelled phosphate due to

autophosphorylation, phosphorylation of tyrosine resdues can be measured using antiphosphotyrosine antibodies. Any suitable method for assessing enzyme activity, as generally known in the art, can be used.

Furthermore, the assays can be adapted to histological methods using suitable labeling reagents applicable to tissue slices and histological specimens. Typical of such reagents are antibodies that are specific for activated as opposed to inactivated forms of enzymes, antibodies specific for phosphotyrosine, and the like. Standard histological procedures can be applied in this context.

Screening Assays

For screening assays, it may be desirable to utilize cells that have been modified to contain specific components of signalling pathways which are used as substrates in the assays of the invention. Any suitable member of a known signalling pathway can be used; an expression system for such a component is transfected into a suitable host cell line for use in these assays. This approach is illustrated herein by transformed Rat1 cell lines modified to contain either the VL30 enhancer-controlled CAT expression system or an expression system for v-src kinase. However, cell lines can be created which contain enhanced amounts of additional components known to be important in signalling pathways such as c-erbB2/her2 or c-ras. Standard recombinant methods are used to obtain these cell lines. The assays are performed using these modified cells in the same manner as described above for assessing the effects of candidates on tumor cells. The methods used to assess the effect of the candidate will be modified, of course, to reflect the nature of the product of the expression system. For example, if the VL30 enhancer-controlled CAT expression system is used, assays which detect CAT activity or CAT-encoding mRNA can be used.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Effect of CAI and SKF96365 on v-src Kinase Activity

The cell line RSV-Rat1, provided by Tom Parsons, University of Virginia Health Sciences Center, Charlottesville, VA, contains an expression vector for the v-src oncogene. The cells were grown at 37°C in 5% CO_2 in DMEM (BioWhittaker) supplemented with 5% bovine calf serum (Hyclone) and gentamicin (10 mg/ml). New cultures were started from frozen stocks every 4-6 weeks.

The cells were grown to confluence in 100 mm plates and were incubated in reduced (0.05%) serum when agonists were added.

After incubation in the presence and absence of drug (usually for 24 hours) plates were washed twice with cold PBS, lysed on ice in M-TG buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES, pH8, 2 mM Na_3VO_4, 150 mM NaCl, 1 mM PMSF, 1% aprotinin). The lysates were cleared by centrifugation and tested for protein content.

Lysates containing 400 µg of protein were precipitated with a pan src monoclonal antibody (α-src, Upstate Biotechnology, Inc.) for 18 hours at 4°C in the presence of protein A agarose. The immune complexes were collected by centrifugation. The pellet was resuspended and kinase activity was measured in 50 µL of kinase buffer (30 mM Tris 7.5, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM PMSF, 0.4% aprotinin) containing 10 µCi of [γ-^{32}P] ATP (6000 Ci/mmole) and 20°C for 10 minutes. The reactions were stopped with 2X Laemmli buffer, boiled for 2 minutes, and proteins were separated on denaturing acrylamide gels. The gels were dried for 1 hour at 80°C and labeled proteins visualized and quantitated by phosphorimager.

As described above, the cells were incubated with 5 μM CAI over a time period of 0-36 hours and aliquots were removed, immunoprecipitated with anti-SRC and tested for kinase activity. The results over time are shown in Figure 1. A doublet of about 57 kD showed decreased band intensity over time; the inhibition was greater than 95% after 24 hours as compared to control. The upper band represents v-src autophosphorylation as verified by immunoblot with the v-src specific antibody, 2-17, obtained from S. Parsons, University of Virginia. The lower band represents either a degradation product or phosphorylation of the immunoglobulin heavy chain.

The foregoing protocol was repeated using various concentrations of SKF96365 in the range of 0-30 μg/ml. Kinase activity was measured as described above. Figure 2 is a histogram showing the concentration-dependent ability of SKF96365 (SKF) to inhibit v-src kinase. As shown in Figure 2, the kinase activity falls significantly below its normal value with concentrations of SKF as low as 0.1 μg/ml. The kinase activity appears reduced to 50% of control at 5-10 μg/ml of SKF and to roughly 10% of its control value at about 30 μg/ml of SKF.

Example 2

Correlation of src Kinase Inhibition with Antiproliferative Effects

The RSV-Rat1 cells of Example 1 were seeded at low density and grown in the presence or absence of CAI. Proliferation was measured directly by cell counts obtained at fixed time intervals. CAI greatly reduced the growth rate as compared to nontreated controls.

The cells were plated at low density in soft agar with and without either CAI or SKF. After 2 weeks at 37°C, anchorage-independent colonies were counted and measured using a gridded ocular. The mean colony size

for cells treated with CAI or SKF was significantly less than controls.

Example 3

Effect of CAI and SKF on p34cdc2

The kinase assays on RSV-Rat1 cells were conducted as described in Example 1 except that immunoprecipitation was conducted using anti-p34cdc2, histone H1 was used as the substrate for phosphorylation, and the kinase reaction was run for 30 minutes. The cells were treated with 5 μM CAI or 5 μM SKF (5 mg/ml) for 4 or 24 hours prior to harvesting. The results are shown in Figure 3.

CAI dramatically decreases the kinase activity at either 4 or 24 hours incubation. After 4 hours, the kinase activity is roughly 50% of control; after 24 hours, the kinase activity is roughly 25% of control. SKF also decreases kinase activity after incubation for either 4 or 24 hours; after 4 hours, the activity is roughly 60% of control; after 24 hours, about 80% of control. Figure 3 shows that this is a statistically significant effect ($P \leq 0.05$) effect. This effect is, however, less dramatic than that at 4 hours.

Example 4

Correlation of p34 Kinase Inhibition with Inhibition of Proliferation

In addition to RSV-Rat1, two additional cell lines, SKOV3 and IOSE, were tested for p34cdc2 kinase activity after treatment with CAI and SKF. SKOV3 is a human ovarian tumor cell line, and IOSE is a partially-transformed cell line derived from human ovarian surface epithelium.

CAI and SKF were able to lower p34cdc2 kinase activity in both SKOV3 and IOSE cell lines. These compounds also inhibited proliferation of these cell

lines as measured by thymidine uptake and as measured by a decreased ability to proliferate in soft agar.

Example 5

Effect of Various Anti-Tumor Compounds on p34 Kinase Activity in Ovarian Tumor Cell Lines

The SKOV3 and IOSE cell lines described in Example 4, plus an additional ovarian tumor cell line designated OVCAR, were exposed to three different compounds: tamoxifen, curcumin, and bryostatin. Tamoxifen is an analog of estrogen, and acts as an inhibitor of estrogen-dependent cell growth in breast tumors and some ovarian tumors. Curcumin is a polyphenolic plant compound which has been shown to have anti-tumor promoting activity in breast, colon, skin, stomach, and duodenum (reviewed in Stoner and Mukhtar, *J Cellular Biochemistry* (1995) **22**:169-180). Bryostatin is an analog of tumor-promoting phorbol esters; depending on the cell type, bryostatin can act as either a mimic of or an inhibitor of the tumor-promoting phorbol esters (see for example Sako *et al. Cancer Research* (1987) **47**:5445-5450). The cultured ovarian cells were exposed to these agents for 24 hours at concentrations known to affect proliferation in sensitive cells (tamoxifen, 500 nM, curcumin, 5 μM, bryostatin, 100 nM), and cell extracts were harvested for use in p34 kinase assays as described in Example 3. Control cells were exposed to the DMSO vehicle for 24 h. The results obtained with each drug paralleled the results obtained in more traditional 3H-thymidine incorporation assays.

Curcumin, which inhibited 3H-thymidine incorporation in all cell lines tested, also inhibited p34-cdc2 kinase activity to less than 50% of control values in each of the cell lines tested -- to 20% of control in OVCAR, 50% in SKOV3, and 45% in IOSE.

The anti-estrogen tamoxifen inhibited p34-cdc2 kinase activity in all three ovarian cell lines, although

the extent of inhibition varied from 10% to 50% -- to 70% of control in OVCAR, 49% in SKOV3, and 50% in IOSE. These differences are likely to reflect differences in the sensitivity of ovarian tumors to tamoxifen therapy.

Bryostatin increased p34-cdc2 kinase activity in SKOV3 cells to 180% of control values, and also stimulated 3H-thymidine incorporation in SKOV3 cells. In IOSE and OVCAR cells, bryostatin had little or no effect on 3H-thymidine incorporation and little or no effect on p34 kinase activity.

These results indicate that p34-cdc2 kinase activity can serve as a good indicator for both inhibitors and stimulators of cell growth.

Example 6

Effect of CAI on Expression in the VL30 Enhancer-Controlled System

A Rat1 cell line modified to contain the TK3R-CAT expression system designed to produce the product of the reporter gene CAT in response to stimulation of the VL30 enhancer element has been described, as noted above, by Rodland, K.D. *et al.* Mol Endocrinol (1993) 7:787-796. This rat cell line, designated TK3R-3, was maintained in DMEM+10% defined calf serum at 37°C in 5% CO_2. Cultures were supplemented with 10 µg/ml gentamicin, 1.75 mg/ml amphotericin B, and 750 µg/ml G418 to maintain selection for the neomycin resistance transfection marker. To conduct the assay, the cells were grown to influence in 12-well plates and were serum deprived in DMEM for 72 hours prior to agonist addition.

To measure CAT activity, cellular proteins were extracted 4 hours after stimulation with agonist in 10 mM Tris HCl, 0.05% Triton X-100 and the extracts were incubated for 10 minutes at 70°C to inhibit cellular acetylases. CAT activity was measured by the assay of Neumann, J.R. *et al.* BioTechniques (1987) 5:444-447, as

previously described by Rodland, K.D. *et al.* (supra). Labeled acetyl CoA incorporation was measured over time and data from the linear portion of the reaction curve were used.

Expression, as measured by CAT activity, was determined in response to a series of known agonists in untreated cells or cells pretreated with CAI for various times before addition of agonist (to assess effects of chronic exposure) or treated with CAI along with agonist (to assess effects of acute exposure).

The agonists tested were thapsigargin (2μM) (thapsi or TG), epidermal growth factor 10 ng/ml (EGF), phorbol acetate (100 ng/ml) (TPA), and combinations of these. The results for the agonists above and for acute exposure to CAI are shown in Figure 4. With respect to agonist activity per se, it is apparent that while neither thapsigargin nor EGF alone stimulates CAT activity significantly, the combination is stimulatory; similarly, the combination of TPA and thapsigargin is more effective than either alone.

In the assay shown in Figure 4, bars marked CAI represent the results when 10 μg/ml CAI was added with the agonist (control cells were exposed to 0.5% DMSO as a vehicular control). As shown in Figure 4, CAI has a stimulatory effect with respect to all of the agonists tested when the cells are exposed to this drug under these "acute" conditions.

The difference in the effect of CAI on the expression of the VL30 reporter system in acute as compared to chronic conditions is evident from the results shown in Figure 5. The KT3R-3 cells were cultured as described above, and 10 μg/ml CAI was added to the cells at various times over the 24 hour period prior to stimulation with 10 μg/ml EGF plus 2 μg/ml thapsi. The cells were harvested 4 hours after addition of (EGF + thapsi) and CAT activity was measured as

described above. As shown in Figure 5, pretreatment with CAI for 0-2 hours resulted in stimulation of expression, while pretreatment for 4 hours or more reduced expression of CAT to less than half of the control value.

Additional assays demonstrated that pretreatment with CAI for 4 or 24 hours completely inhibited the ability of thapsigargin to stimulate TK3R-CAT gene expression in conjunction with either EGF, TPA or cAMP. However, the ability of EGF, TPA and cAMP to act synergistically in the absence of thapsigargin was uneffected. Similar results were obtained following a 24-hour pretreatment with SKF.

The effects of the CAI and SKF under chronic exposure conditions are summarized in Figures 6A and 6B. The assays were conducted as described above; EGF was added at 10 µg/ml, thapsi (designated TG in this figure) at 2 µg/ml and TPA at 100 ng/ml. In Figure 6A, showing the results for CAI, the light bars show the effects of agonists in the absence of CAI and the dark bars, the comparative levels of expression when 10 µg/ml CAI was added 24 hours prior to the designated agonist. Similarly, in Figure 6B, the light bars show the stimulation effected by the designated agonist, and the dark bars the stimulation after a 24 hour pretreatment with 10 µg/ml SKF. In both instances, inhibition of the agonist activity was observed for the agonists of EGF/TG, TPA, and TPA/TG; however, even under chronic conditions, slight stimulation was observed with the combination agonist of EGF/TPA.

These results indicate that inhibition of VL30 enhancer-driven expression can be used to predict likely stimulators or inhibitors of cell proliferation. Furthermore, strategic comparisons of the effect of the drug on various known inducers of VL30 enhancer expression can provide useful information about the specific signalling pathways affected by the drug.

Claims

1. A method to screen a candidate drug for its ability to inhibit cell proliferation through interaction with intracellular signal pathways in target cells, which method comprises

incubating a test sample of said cells in the presence of said candidate and incubating a control sample of said cells in the absence of said candidate;

measuring the activity of p34cdc2 kinase in each of said cell samples;

comparing the activities of said p34cdc2 kinase in said cell samples;

wherein a diminution of activity of said kinase in the presence of the candidate as compared to the absence of the candidate indicates the ability of the candidate to inhibit proliferation.

2. The method of claim 1 wherein the activity of said kinase is measured by precipitating p34cdc2 kinase from an extract of said cell samples with anti-p34 antibody; and

assaying the phosphorylation of a substrate by the precipitated kinase.

3. The method of claim 2 wherein said assaying is conducted by determining the incorporation of radiolabeled phosphate from ATP into the substrate.

4. The method of claim 2 wherein the substrate is histone H1.

5. The method of claim 1 wherein said measuring is conducted by reacting an extract or a histologic sample of the cells with an antibody specific for activated as opposed to inactivated p34cdc2 kinase and measuring the amount of complex formed between the

antibody and components of the extract or the histologic sample.

6. The method of claim 1 wherein said incubating is conducted for sufficient time to permit the candidate to interact with p34cdc2 kinase.

7. The method of claim 1 wherein said cells have been modified to contain said p34cdc2 kinase.

8. A method to screen a candidate drug for the ability to function as an antitumor agent with respect to target tumor cells, which method comprises

incubating a test sample of said cells in the presence of said candidate and incubating a control sample of said cells in the absence of said candidate;

measuring the activity of p34cdc2 kinase in each of said cell samples;

comparing the activities of said p34cdc2 kinase in said cell samples;

wherein a diminution of activity of said kinase in the presence of the candidate as compared to the absence of the candidate indicates the ability of the candidate to function as an antitumor agent.

9. A method to predict whether a tumor will or will not respond to treatment with a drug that is an inhibitor of cell proliferation signal pathways, which method comprises

obtaining a biopsy of said tumor;

incubating a test portion of said biopsy in the presence of said drug and incubating a control portion of said biopsy in the absence of said drug;

measuring the activity of p34cdc2 kinase in the portion incubated in the presence of the drug and of the portion incubated in the absence of the drug;

comparing the activity of the p34cdc2 kinase in each of said portions;

whereby a diminution in the activity of the kinase in the presence as opposed to the absence of the drug indicates that the tumor will respond to the drug.

10. A method to predict whether a tumor will or will not respond to the drug CAI or SKF96365 which method comprises

obtaining a biopsy of said tumor;

incubating a test portion of said biopsy in the presence of said drug and incubating a control sample of said biopsy in the absence of said drug;

measuring the activity of src kinase in each of said portions;

comparing the activities of said src kinase in said portions;

wherein a diminution of activity of said kinase in the presence of the drug as compared to the absence of the drug indicates that the tumor will respond to the drug.

11. A method to screen candidate drugs for their ability to stimulate or to inhibit cell proliferation through interaction with intracellular signal pathways in target cells, wherein said target cells contain the VL30 reporter expression system, which method comprises

incubating a test sample of said cells in the presence of said candidate and incubating a control sample of said cells in the absence of said candidate under either acute or chronic conditions;

treating said cells with at least one agonist to effect the expression mediated by the VL30 reporter system in each of said cell samples;

measuring the expression mediated by the VL30 reporter system in each of said cell samples;

comparing the expression of said VL30 reporter system in said cell samples;

wherein an increase of said expression in the presence of the candidate as compared to the absence of the candidate indicates the ability of the candidate to stimulate proliferation under said acute or chronic conditions, and a decrease of said expression in the presence of the candidate as compared to the absence of the candidate indicates the ability of the candidate to inhibit proliferation under said acute or chronic conditions.

12. The method of claim 11 wherein said cells have been stably modified to contain said VL30 reporter system.

13. The method of claim 11 wherein said chronic conditions consist essentially of pretreating said cells with the candidate for 4-24 hours of prior to treating such cells with the agonist.

14. The method of claim 11 wherein said acute conditions consist essentially of treating the cells with candidate simultaneously with treating the cells with agonist.

15. The method of claim 11 where the VL30 reporter system expresses the product of the CAT gene.

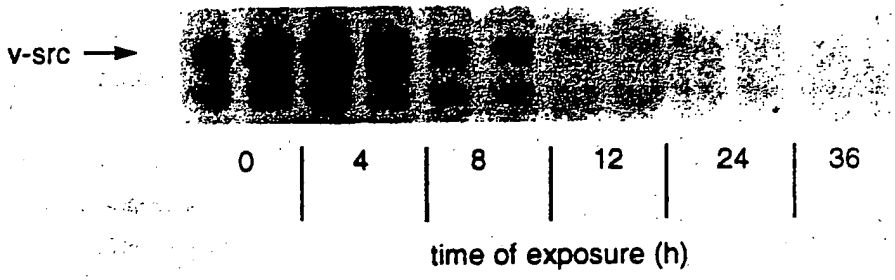

FIG. 1

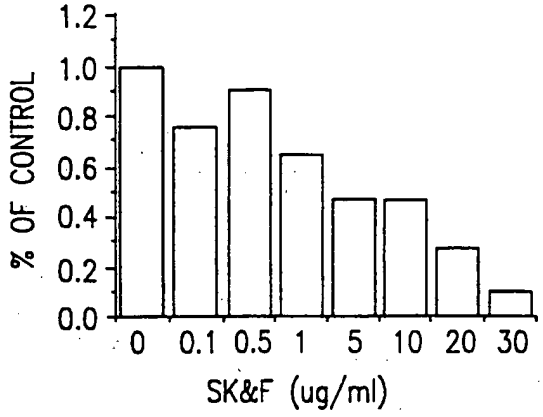

FIG. 2

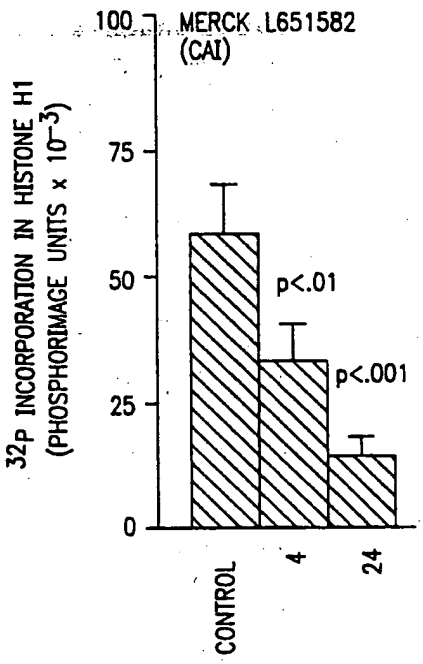

FIG. 3A

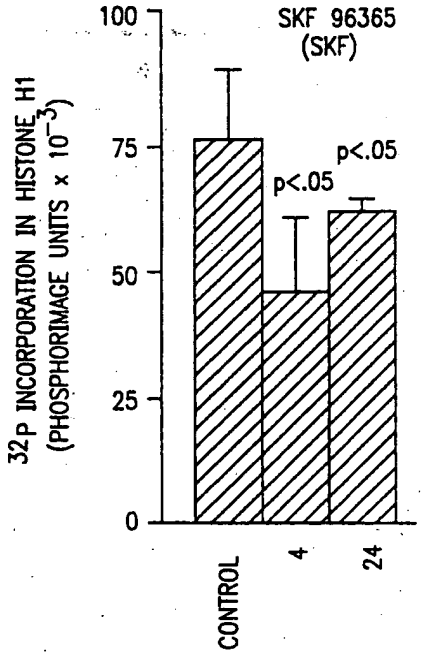

FIG. 3B

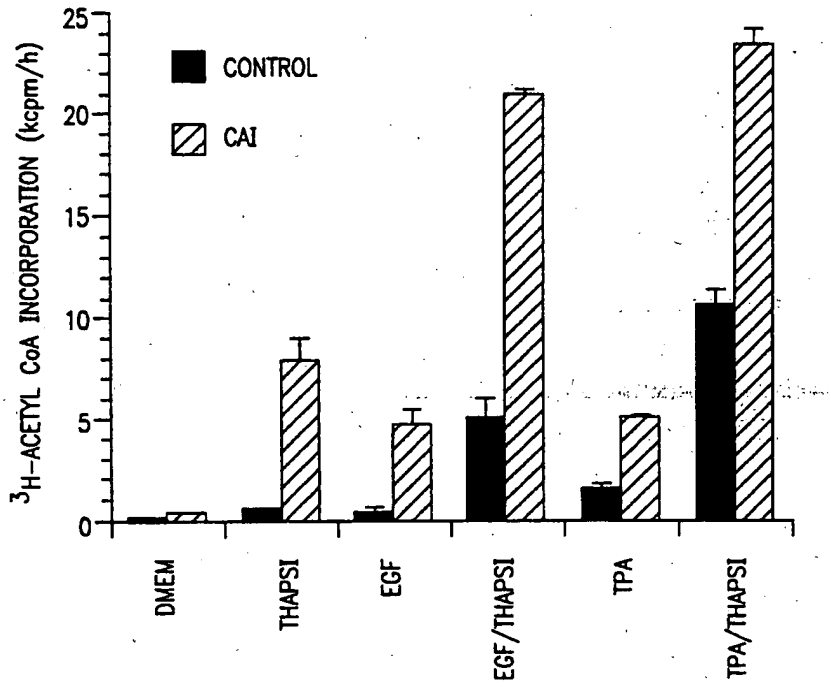
^{3}H-ACETYL CoA INCORPORATION (kcpm/h)
25
20
15
10
5
0
CONTROL
CAI
DMEM
THAPSI
EGF
EGF/THAPSI
TPA
TPA/THAPSI

FIG. 4

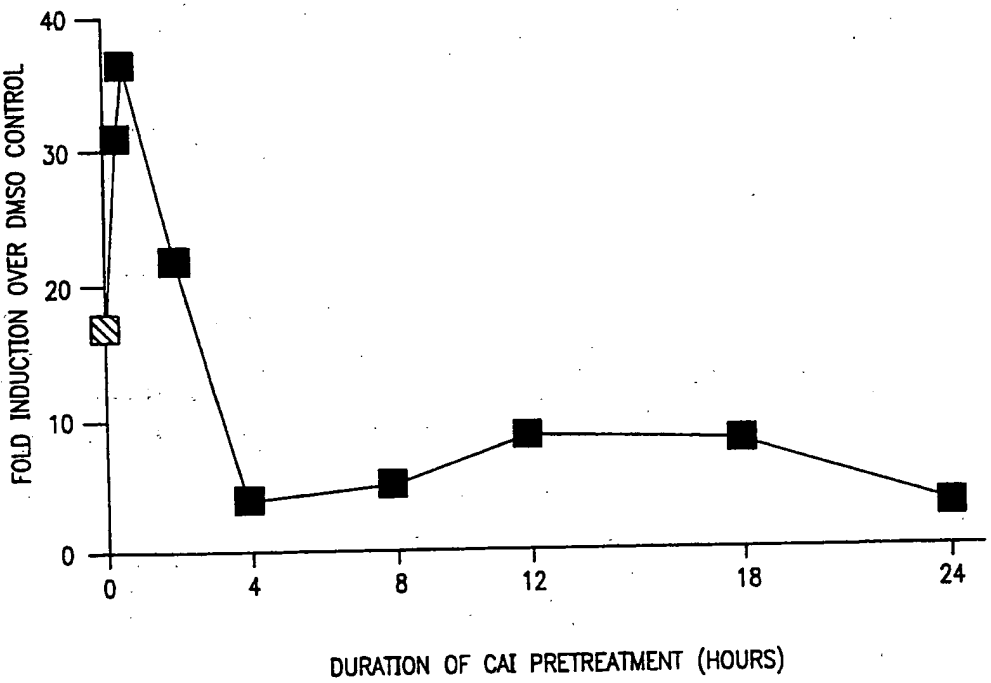
40
30
20
10
0
FOLD INDUCTION OVER DMSO CONTROL
0
4
8
12
18
24
DURATION OF CAI PRETREATMENT (HOURS)

FIG. 5

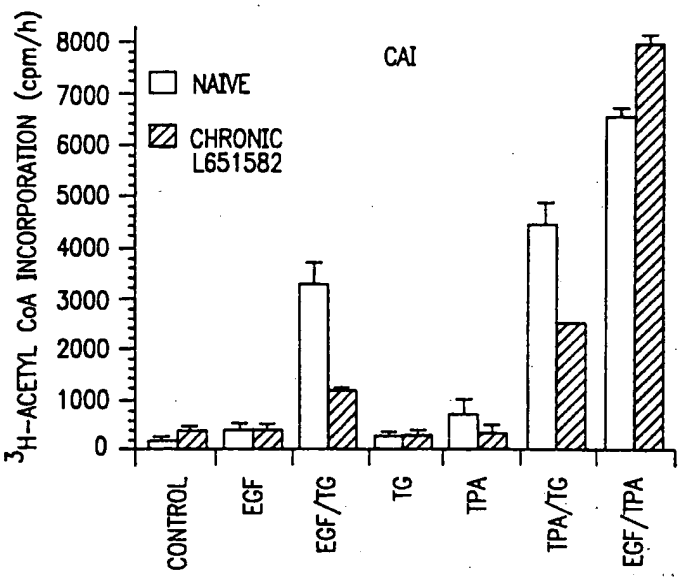
CAI
NAIVE
CHRONIC L651582
^{3}H-ACETYL CoA INCORPORATION (cpm/h)
8000
7000
6000
5000
4000
3000
2000
1000
0
CONTROL
EGF
EGF/TG
TG
TPA
TPA/TG
EGF/TPA

FIG. 6A

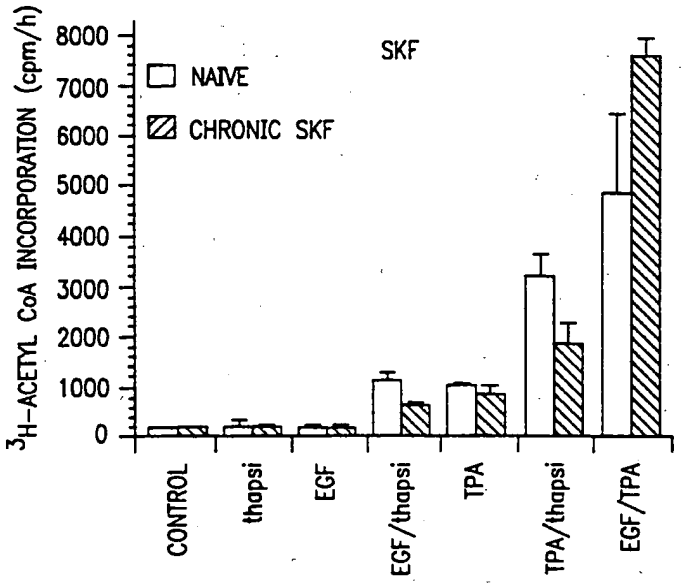
SKF
NAIVE
CHRONIC SKF
^{3}H-ACETYL CoA INCORPORATION (cpm/h)
8000
7000
6000
5000
4000
3000
2000
1000
0
CONTROL
thapsi
EGF
EGF/thapsi
TPA
TPA/thapsi
EGF/TPA

FIG. 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/11330

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/00; G01N 33/574
US CL :435/4, 7.23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN:Medline, Biosis, CAPlus, WPIDS search terams p34cdc2 kinase, cdc2 kinase, src kinase, vl30

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/17413 A1 (PARACELSIAN, INC.) 04 August 1994, pages 19-21, 24, 25.	1-9
Y	GALLICK et al. The effects of biologic response modifiers on c-src kinase activity in human colorectal carcinoma cell lines. Journal Cell Biochem. Suppl. 1990, Vol.14 part B, page 24, see entire document.	10
X -- Y	NILSSON et al. Inducible and cell type-specific expression of VL30 U3 subgroups correlate with their enhancer design. Journal Virol. January 1994, Vol.68, pages 276-288, see entire document.	11, 13-15 --- 12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 12 SEPTEMBER 1996	Date of mailing of the international search report 02 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Freese /by ROBERT A. SCHWARTZMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/11330

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAGUN et al. Transient inhibition of protein synthesis induces the immediate early gene VL30: alternative mechanism for thapsigargin-induced gene expression. Cell Growth Differentiation. July 1995, Vol.6, pages 891-897.	11-15